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### Neutral and Nonneutral Genetic Markers Revealed the Presence of Inshore and Offshore Stock Components of Atlantic Cod in Greenland Waters

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ARTICLE

# Neutral and Nonneutral Genetic Markers Revealed the Presence of Inshore and Offshore Stock Components of Atlantic Cod in Greenland Waters

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## Abstract

The spawning stock of Atlantic cod *Gadus morhua* in West Greenland waters was characterized by a drastic decline in the late 1960s and has since exhibited considerable variation. It has been suggested that the cod stock in West Greenland waters is composed of several stock components that include (1) a number of distinct local inshore populations spawning in separate fjord systems, (2) an offshore spawning component located on the fishing banks, and (3) a periodic Icelandic–East Greenland cod influx that mixes with the offshore and inshore West Greenland stock components. In an attempt to clarify the status of Atlantic cod in Greenland waters, we investigated the genetic structure at different inshore and offshore feeding grounds east and west of Greenland. A total of 1,581 genetic samples were collected within North Atlantic Fisheries Organization areas at inshore and offshore locations as well as within the International Council for the Exploration of the Sea area XIVb. Those samples were genotyped for 18 microsatellite loci and the pantophysin (*Pan I*) locus. Both types of genetic markers gave congruent results and suggest the presence of two distinct genetic components with limited connectivity in Greenland waters, namely, an inshore component and an offshore component.

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During the last century, Atlantic cod *Gadus morhua* stocks declined dramatically in several regions owing to drastic overexploitation and environmental changes (Buch et al. 1994; Christensen et al. 2003). The depletion of those cod stocks was primarily due to the failure of sustainable fishery management and the mismatch between management and biological units (Stephenson and Kenchington 2000; Reiss et al. 2009).

Historically, the commercial fishery for the West Greenland Atlantic cod stock began in 1911 in local fjords where cod could be caught on a regular basis during summer and autumn (Horsted 2000). In the late 1920s, an offshore fishery was initiated and landings reached a peak of 350,000–450,000 metric tons. A minor part of these catches originated from the East Greenland offshore area (see Storr-Paulsen et al. 2004 for a detailed

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description). The Atlantic cod stock in offshore waters of West Greenland drastically declined in the last century and was near extinction in the late 1960s owing to environmental changes and a heavy exploitation (Buch et al. 1994). A large incoming Icelandic year-class, believed to be that of 1984 (Schopka 1993), increased the stock's abundance, resulting in offshore catches of about 70,000 metric tons in 1989. Since then no offshore fishery has existed, and only a small inshore fishery with a total catch of about 5,000 metric tons remained in later years (Wieland and Storr-Paulsen 2005). In a 12-year period, no offshore cod fishery existed in West Greenland owing to the depletion of this stock component. However, in later years, there have been indications of increasing abundance of cod in the offshore waters owing to new incoming year-classes, probably of East Greenland or Iceland origin (Wieland and Storr-Paulsen 2005; ICES 2010), and a small fishery has been initiated in West Greenland offshore waters with a total catch of about 5,000 metric tons in 2009 (Wieland and Storr-Paulsen 2005; ICES 2010). In the same time period a small-scale fishery in the inshore area has existed and, similar to the offshore area, an increase in the abundance has been evident in recent years. Although similar good year-classes can be found in all waters, the inshore area in later years have experienced relatively good year-classes not found in the offshore area (ICES 2010). Tagging experiments carried out at East Greenland showed that cod tagged in the area were usually recaptured at Icelandic spawning grounds and rarely at West Greenland spawning grounds (Riget and Hovgård 1989). At present, the Atlantic cod stock in Greenland waters is managed as two different units (ICES 1996): an offshore unit composed of East and West Greenland offshore populations, and an inshore unit comprising inshore populations from waters off West Greenland (North Atlantic Fisheries Organization [NAFO] Area 1B and 1D) (ICES 2007).

Recently, microsatellite markers have frequently been used in an attempt to resolve uncertainties related to stock structure within management regions in Atlantic cod. Consequently, subtle but biologically meaningful genetic differences among populations within management regions across the Atlantic were revealed (Bentzen et al. 1996; Ruzzante et al. 1996; Hutchinson et al. 2001; Beacham et al. 2002; Knutsen et al. 2003; Pampoulie et al. 2006). In addition, nonneutral markers, such as the pantoophysin (*Pan I*) locus, which has proved to be useful in the discrimination of populations of Atlantic cod (Fevolden and Pogson 1997; Case et al. 2005; Sarvas and Fevolden 2005a, 2005b; Pampoulie et al. 2006; Skarstein et al. 2007), have increasingly been used to detect differences among recently separated populations that might not be revealed by neutral markers alone.

Although several biological and tagging studies have suggested the need for genetic analyses of Greenlandic cod, very few such data existed before this study. The genetic information that was available showed that the frequency of the hemoglobin *HbI<sup>f</sup>* allele was homogeneous among West Greenland samples, while the transferrin allele *Tf<sup>C</sup>* exhibited a lower frequency in West Greenland samples than in Cape Farewell, south Greenland (de Ligny 1969).

Hence, we describe for the first time the genetic structure of Atlantic cod around Greenland. We examined the genetic variation of 18 expected neutral microsatellite loci and the *Pan I* locus, known to be under selection, among 1,581 adult Atlantic cod collected over a period of 4 years (from 2003 to 2006). Conventional genetic stock identification analyses were performed and the results were interpreted in light of fisheries management objectives.

## METHODS

**Sampling.**—Owing to the absence of spawning in West Greenland offshore waters during the last decade, samples of Atlantic cod from 19 feeding locations were collected ( $n = 1,581$ ) in different NAFO areas in West Greenland waters from 2003 to 2005 and from the International Council for the Exploration of the Sea [ICES] region XIVb in East Greenland in 2004 and 2006 (Figure 1; see Table 1 for sample codes). Several of these locations were sampled in several years (see Table 1; Figure 1). Samples from West Greenland were collected during the summer (June to August, samples 1–12) or in the autumn 2006 (samples 17–19). Samples from East Greenland (samples 13–16) were also collected during the autumn 2006. Biological information such as total length (TL; mm) and maturity stages were collected for each individual.

**DNA methods.**—Gill filaments or fin clips were preserved in 1 mL of 96% ethanol, and DNA was extracted by means of a Chelex (Biorad 10%) extraction protocol (Walsh et al. 1991). Samples were genotyped at 18 polymorphic microsatellite loci: *Gmo2* (Brooker et al. 1994), *Gmo8*, *Gmo19*, *Gmo34*, *Gmo37* (Miller et al. 2000), *Tch5*, *Tch11*, *Tch14*, *Tch22* (O'Reilly et al. 2000), *PGmo38*, *PGmo49* (Jakobsdóttir et al. 2006), and *PGmo61*, *PGmo74*, *PGmo87*, *PGmo94*, *PGmo100*, *PGmo124*, and *PGmo134* (Skirnisdóttir et al. 2008). Polymerase chain reaction (PCR) conditions followed the method of Pampoulie et al. (2006) for the first set of markers (*Gmo8*, *Gmo19*, *Gmo34*, *Gmo37*, *Tch5*, *Tch11*, *Tch14*, and *Tch22*) and of Skirnisdóttir et al. (2008) for the second set (*PGmo38*, *PGmo49*, *PGmo61*, *PGmo74*, *PGmo87*, *PGmo94*, *PGmo100*, *PGmo124*, and *PGmo134*). Samples were then analyzed on an ABI PRISM 3730 sequencer with the GeneScan-500 LIZ size standard and genotyping was performed with GeneMapper version 3.5 (Applied Biosystems, Foster City, California).

Samples were also genotyped at the *Pan I* locus. We used a recently published method that involves a fluorescent allele-specific duplex PCR (Stenvik et al. 2006). This method is essentially based on the description of new primer sequences (allele-specific nucleotides in bold and PIG-tail sequence underlined) that facilitate the amplification of the *Pan I* locus by removing the *DraI* digestion procedure. Universal primers were defined by Stenvik et al. (2006): universal forward PIG-tail (*PanI*-2-PIG): 5'-TTTTCTTTGACAGCGCTTG-GCAAATGAA, reverse *Pan I*<sup>A</sup>-specific 5'-6FAM labeled (*PanI*-5): 5'-GCTTAAGCAGATATCGCAGTAGTTTC, reverse *Pan I*<sup>B</sup>-specific 5'-PET labeled (*PanI*-6): 5'-

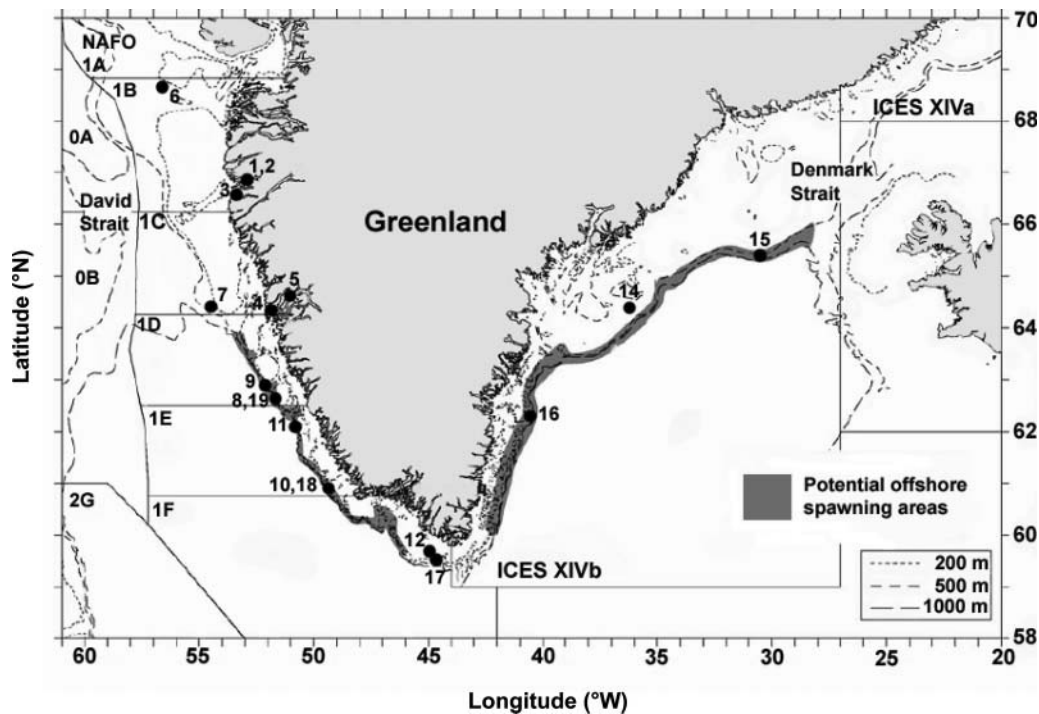


FIGURE 1. Locations at which Atlantic cod samples were collected in Greenland waters from 2003 to 2006. See Table 1 for additional information. Sample 13 (from area XIVb in 2004) is not shown. The figure is modified from Storr-Paulsen et al. 2004. At present, the cod stock in Greenland is managed as two different units, an offshore unit composed of East and West Greenland offshore populations (areas XIVb, 1E, and 1F) and an inshore unit composed of West Greenland inshore populations (areas 1B and 1D).

TTAAGCAGATCTCGCAGTAGTTTT. The PCR cycle conditions for this marker were 94°C for 4 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 50 s, and then a final extension at 72°C for 7 min.

### Statistical Methods

**Marker neutrality.**—The assumption of selective neutrality remains crucial for the conclusion drawn from data obtained at microsatellite loci. Therefore, we applied the coalescent-based simulation methods of Beaumont and Nichols (1996) implemented in the software FDIST2. The software calculated the level of differentiation as  $F_{ST}$  values and heterozygosity for each locus according to Weir and Cockerham (1984) and expected  $F_{ST}$  values from the data, weighted by their heterozygosity. Coalescent simulations were then performed with samples of the same size as the observed samples and assuming an island model of 100 islands. One hundred thousand independent loci were generated with the infinite allele mutation model. Simulated distribution of  $F_{ST}$  values conditional on heterozygosity under a neutral model were thus obtained and compared with the observed  $F_{ST}$  values to identify potential outliers loci.

**Genetic variability within the collected samples.**—Allele frequencies, observed heterozygosity ( $H_o$ ), and unbiased expected heterozygosity ( $H_e$ ) were calculated in GENETIX version 4.03 (Belkhir et al. 2002). Tests of Hardy-Weinberg

equilibrium (HWE) were performed with Fisher exact tests in GENEPOP'007 (Rousset 2008). An analysis of genetic linkage disequilibrium between pairs of loci was performed with the log-likelihood ratio statistic implemented in the same software.

**Differentiation among samples.**— $F$ -statistics (Wright 1969) were calculated from allele frequencies at all loci examined for each population according to Weir and Cockerham's (1984) method with GENETIX. Significance of  $F_{ST}$  estimates was assessed with exact tests implemented in GENEPOP (multilocus and pairwise estimates). Significance levels were adjusted with sequential Bonferroni correction (Rice 1989). To accurately compare the level of genetic divergence detected with highly polymorphic microsatellite loci and the *Pan I* locus, the standardized genetic differentiation measure ( $G'_{ST}$ ) developed by Hedrick (2005), which is expected to allow comparisons between loci with different level of genetic variation, was calculated.  $G'_{ST}$  represents the proportion of the maximum differentiation possible for the level of subpopulation homozygosity observed and is defined as

$$G'_{ST} = \frac{G_{ST}(1 + H_S)}{(1 - H_S)}.$$

TABLE 1. Sample codes, region of sampling, year and season of sampling, total length, number of individuals scored ( $n$ ), observed heterozygosity ( $H_O$ ), expected heterozygosity ( $H_E$ ), allelic richness ( $A_R$ ; based on 33 individuals); and mean number of alleles (MnA) from 12 samples of Atlantic cod from the West Greenland waters. The *Pan I*<sup>A</sup> allele frequency is reported in the column labelled “%A”. Bold italics indicate departure from Hardy–Weinberg expectations after correction for multiple tests ( $P = 0.00044$ ).

Sample code	Region	Year	Season	Length (mm [mean ± SD])	<i>n</i>	Microsatellite loci				<i>n</i>	<i>Pan I</i> locus		
						<i>H<sub>O</sub></i>	<i>H<sub>E</sub></i>	MnA	<i>A<sub>R</sub></i>		<i>H<sub>O</sub></i>	<i>H<sub>E</sub></i>	%A
Inshore													
1	West	2003	Summer	39.19 ± 9.12	102	0.811	0.839	18.44	14.03	104	0.221	0.267	84
2	West	2004	Summer	38.93 ± 10.48	83	0.810	0.826	19.17	14.60	69	0.406	0.500	51
3	West	2005	Summer	37.90 ± 15.06	94	<b>0.800</b>	0.825	18.78	14.33	92	0.446	0.469	63
4	West	2004	Summer	40.86 ± 4.85	64	0.815	0.830	17.39	14.41	59	0.509	0.437	68
5	West	2005	Summer	32.31 ± 11.74	94	<b>0.804</b>	0.836	19.56	14.65	86	0.372	0.493	86
Offshore													
6	West	2005	Summer	30.14 ± 8.60	94	0.803	0.826	17.72	15.04	50	0.260	0.442	33
7	West	2005	Summer	31.40 ± 7.62	90	0.789	0.815	20.61	15.07	84	0.167	0.210	12
8	West	2004	Summer	31.46 ± 5.28	57	0.778	0.810	16.22	13.94	56	0.304	0.258	15
9	West	2005	Summer	28.57 ± 6.12	94	0.779	0.815	18.94	14.54	84	0.341	0.327	21
10	West	2004	Summer	52.46 ± 7.05	38	0.804	0.811	15.78	15.22	36	0.472	0.413	29
11	West	2005	Summer	26.86 ± 5.64	91	0.787	0.816	19.28	14.56	84	0.333	0.293	18
12	West	2005	Summer	35.04 ± 10.04	94	0.786	0.815	19.00	14.29	89	0.360	0.336	21
13	East	2004	Autumn	67.38 ± 21.39	33	0.790	0.817	15.23	15.22	47	0.277	0.296	18
14	East	2006	Autumn	65.28 ± 18.32	114	<b>0.798</b>	0.818	20.06	14.36	114	0.419	0.474	29
15	East	2006	Autumn	66.07 ± 11.10	61	0.785	0.808	17.44	14.44	48	0.250	0.219	13
16	East	2006	Autumn	40.67 ± 10.00	100	0.789	0.816	19.61	14.53	98	0.245	0.215	12
17	West	2006	Autumn	38.80 ± 8.26	94	0.796	0.811	19.28	14.45	87	0.437	0.400	28
18	West	2006	Autumn	38.15 ± 10.02	98	0.795	0.812	20.06	14.71	93	0.366	0.312	19
19	West	2006	Autumn	32.60 ± 10.66	86	0.786	0.812	19.33	14.95	84	0.250	0.253	15

where  $G_{ST}$  is the index of genetic differentiation and  $H_s$  is the homozygosity of the subpopulation.

The level of genetic differentiation among samples was further investigated by means of a factorial correspondence analysis (FCA) with GENETIX at the microsatellite loci. The factorial analysis is performed on contingency tables in which populations are described by their allele frequencies. The eigenvalues associated with the factorial axes are analogous to partial  $F_{ST}$  estimates, and the ordination of samples along the factorial axes therefore represents the genetic similarity between populations in a three-dimensional space.

To estimate the potential number of populations of Atlantic cod collected in Greenlandic waters, we used the software STRUCTURE (Pritchard et al. 2000). STRUCTURE is a model-based Bayesian, Markov chain–Monte Carlo approach that clusters individuals to minimize Hardy–Weinberg disequilibrium and gametic phase disequilibrium between loci within groups. The posterior distribution of individual admixture proportions ( $q$ ) of individuals was estimated by assuming an admixture model without any foregone assumptions on the population structure. We used a burn-in period of 350,000 steps and 650,000 Markov chain–Monte Carlo simulations. The number of popu-

lations represented in our samples was estimated from  $K = 1$  to  $K = 7$ . Several runs were carried out to estimates convergence of the admixture proportions ( $q$ ).

A locus-by-locus analysis of molecular variance (AMOVA) was carried out in ARLEQUIN version 3.11 (Excoffier et al. 2005) to assess the hierarchical partitioning of genetic variability among regions post hoc defined using the detected genetic pattern during the STRUCTURE runs. A hierarchical AMOVA was performed on the *Pan I* data with the same software.

For both types of genetic marker, a comparison of the level of genetic diversity among inshore and offshore components was performed using the software FSTAT (Goudet 1995).

## RESULTS

Biological information is presented in Table 1.

### Genetic Variability within the Collected Samples

The number of alleles per locus across all samples ranged from 12 (*Tch22*) to 53 (*PGmo134*) (Table 2). Per sample  $H_O$  averaged over all loci ranged from 0.778 to 0.815, while the  $H_E$  per sample ranged from 0.810 to 0.839 (Table 1). Genotypic proportions were out of HWE in only 9 out of 342 exact tests

TABLE 2. Nucleotide repeat characteristics ( $n_r$ ), allelic range ( $R$ ), number of allele ( $n_A$ ), observed heterozygosity ( $H_O$ ) and expected heterozygosity ( $H_E$ ) at 18 microsatellite loci from Atlantic cod.

Locus	$n_r$	$R$	$n_A$	$H_O$	$H_E$
<i>PGmo38</i>	Di-	92–150	30	0.895	0.906
<i>PGmo49</i>	Di-	110–178	24	0.738	0.776
<i>PGmo61</i>	Di-	262–364	39	0.734	0.742
<i>PGmo74</i>	Hepta-	269–479	26	0.836	0.875
<i>PGmo87</i>	Di-	185–249	30	0.847	0.865
<i>PGmo94</i>	Di-	268–366	34	0.790	0.828
<i>PGmo100</i>	Tri-	217–256	14	0.762	0.772
<i>PGmo124</i>	Di-	152–260	42	0.877	0.903
<i>PGmo134</i>	Di-	216–364	53	0.916	0.920
<i>Gmo2</i>	Di-	109–153	23	0.780	0.851
<i>Gmo8</i>	Tetra-	112–324	51	0.930	0.931
<i>Gmo19</i>	Tetra-	131–267	31	0.846	0.922
<i>Gmo34</i>	Tetra-	80–140	14	0.311	0.338
<i>Gmo37</i>	Tetra-	238–362	21	0.842	0.853
<i>Tch5</i>	Tetra-	176–288	27	0.922	0.928
<i>Tch11</i>	Tetra-	133–237	27	0.882	0.919
<i>Tch14</i>	Tetra-	81–281	43	0.938	0.952
<i>Tch22</i>	Tetra-	76–120	12	0.604	0.609

after Bonferroni correction for multiple tests (25 were out of HWE before correction) and were not due to any specific loci (no technical artifact).

All samples but three (samples 3, 5, and 14) exhibited HWE (Table 1). Genotype data were also tested for linkage disequilibrium and 24 comparisons between pairs of loci in different populations remained significant after correction for multiple

tests. None of these comparisons were due to any particular loci or populations, thus suggesting that the results were not due to physical linkage of the studied loci.

The  $H_o$  per sample at the *Pan I* locus ranged from 0.167 to 0.509, while  $H_e$  ranged from 0.210 to 0.500 (Table 1). There was no evidence for departure from HWE for any of the samples (Table 1).

### Neutrality of the Markers

Simulation tests for selection suggested that the variation at microsatellite loci departed significantly from neutral expectations at *Gmo34* ( $P = 0.032$ ; Figure 2). Observed  $F_{ST}$  for this locus exceeded the 99% confidence interval of the simulated distribution. The *Pan I* locus was an extreme outlier, with a  $F_{ST}$  value ( $F_{ST} = 0.254$ ), 10 times larger than that expected for a neutral locus.

### Differentiation among Samples at the Microsatellite Loci

The partitioning of genetic variance based on microsatellite loci among and within the 19 samples, as estimated by  $F$ -statistics, showed a mean significant  $F_{ST}$  value of 0.002 and a  $F_{IS}$  (inbreeding coefficient) of 0.035. After sequential Bonferroni correction, pairwise differentiation between populations yielded 53 significant comparisons out of 171 (101 were significant before correction), of which 48 out of 75 (66 before correction) were due to the comparisons among inshore and offshore samples (Table 3). Pairwise comparison of the temporal samples revealed temporal stability in all replicates.

A clearer and more detailed pattern of the genetic variation among populations was revealed by FCA. The first three factors explained 25.98% of the total inertia (factor 1 = 11.48%, eigenvalue = 0.024; factor 2 = 7.81%, eigenvalue = 0.016; factor 3 = 6.70%, eigenvalue = 0.014). Although variation could be

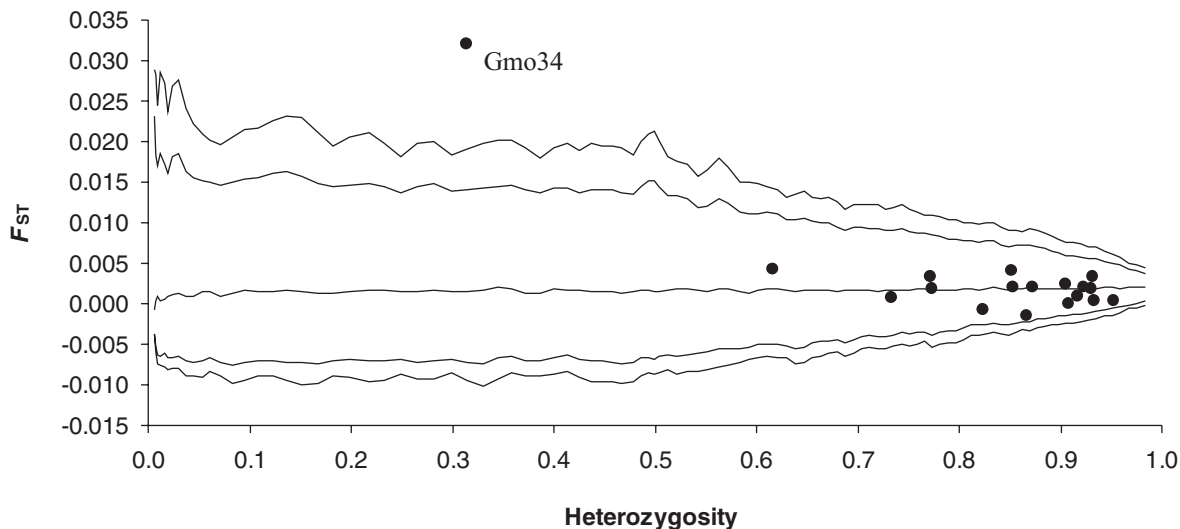


FIGURE 2. Simulated distribution of the level of genetic differentiation ( $F_{ST}$ ) and heterozygosity in Atlantic cod under the infinite alleles model using the weighted heterozygosity for assumed neutral loci. The median is enclosed by the 95% (solid lines) and 99% (dashed lines) confidence limits of  $F_{ST}$ . The  $F_{ST}$  value of the *Pan I* locus was 0.254.

TABLE 3. Estimates of  $F_{ST}$  among pairs of samples of Atlantic cod in Greenland waters. Below the diagonal are estimates for 18 microsatellite loci; above the diagonal are estimates for the *Pun I* locus. Underlined values were significant after sequential Bonferroni correction.

Pop	Inshore					Offshore														19
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
1	0	0.231	0.110	0.069	0.173	0.442	0.682	0.641	0.576	0.494	0.610	0.567	0.244	0.126	0.316	0.351	0.146	0.245	0.304	
2	0.004	0	0.021	0.05	-0.003	0.052	0.301	0.237	0.178	0.079	0.213	0.169	0.392	0.249	0.470	0.506	0.276	0.391	0.456	
3	0.002	0.001	0	-0.002	0.003	0.152	0.422	0.361	0.301	0.189	0.337	0.292	0.609	0.458	0.668	0.680	0.491	0.591	0.646	
4	0.001	0.002	0.001	0	0.022	0.208	0.504	0.438	0.371	0.252	0.411	0.361	0.319	0.191	0.390	0.425	0.215	0.320	0.379	
5	0.004	0.003	0.003	0.001	0	0.090	0.348	0.286	0.227	0.122	0.263	0.218	0.195	0.083	0.268	0.304	0.101	0.195	0.255	
6	0.002	0.001	0.001	0.001	0.002	0	0.121	0.074	0.031	-0.011	0.053	0.026	-0.005	0.014	0.019	0.025	0.005	-0.004	0.008	
7	0.006	0.002	0.002	0.004	0.003	0.001	0	-0.003	0.021	0.090	0.008	0.026	-0.008	0.033	0.004	0.008	0.021	-0.004	-0.002	
8	0.006	0.001	0.001	0.003	0.005	0.001	-0.001	0	0.003	0.049	-0.004	0.006	0.044	-0.005	0.101	0.121	-0.002	0.042	0.083	
9	0.007	0.003	0.003	0.002	0.004	0.003	0.001	0.001	0	0.011	-0.003	-0.005	0.002	-0.008	0.075	0.089	-0.008	0.020	0.055	
10	0.009	0.002	0.005	0.003	0.006	0.004	0.001	0.001	0.002	0	0.029	0.000	0.006	0.084	-0.009	-0.006	0.069	0.015	-0.003	
11	0.007	0.002	0.003	0.002	0.004	0.001	-0.001	-0.001	0.001	0.002	0	-0.004	-0.006	0.017	0.015	0.020	0.008	-0.005	0.003	
12	0.007	0.003	0.003	0.003	0.004	0.002	-0.001	0.001	0.001	0.001	-0.001	0	-0.006	0.050	-0.005	-0.002	0.037	-0.001	-0.007	
13	0.007	0.003	0.004	0.003	0.003	0.004	0.002	0.003	0.002	0.001	0.001	0.001	0	0.028	0.002	0.0067	0.017	-0.007	-0.005	
14	0.006	0.001	0.002	0.002	0.003	0.001	0.001	0.001	-0.001	0.004	0.001	0.001	0.001	0	0.071	0.083	-0.003	0.025	0.056	
15	0.006	0.002	0.002	0.002	0.005	0.001	-0.001	0.001	0.001	0.002	-0.001	-0.001	0.001	-0.001	0	-0.007	0.057	0.010	-0.006	
16	0.008	0.002	0.003	0.003	0.003	0.003	0.001	0.001	-0.001	0.002	0.001	0.001	-0.001	0.001	0.001	0	0.068	0.015	-0.002	
17	0.005	0.001	0.002	0.002	0.003	0.001	0.001	0.002	-0.001	0.002	-0.001	0.001	0.001	-0.001	-0.001	0.001	0	0.014	0.042	
18	0.007	0.002	0.004	0.004	0.005	0.002	0.001	0.001	0.001	0.001	0.001	0.0003	0.003	0.001	0.001	0.001	0.003	0	0.002	
19	0.069	0.002	0.002	0.002	0.003	0.001	0.001	0.001	0.001	0.001	-0.001	-0.001	0.001	0.001	0.001	0.001	-0.001	0.0012	0	



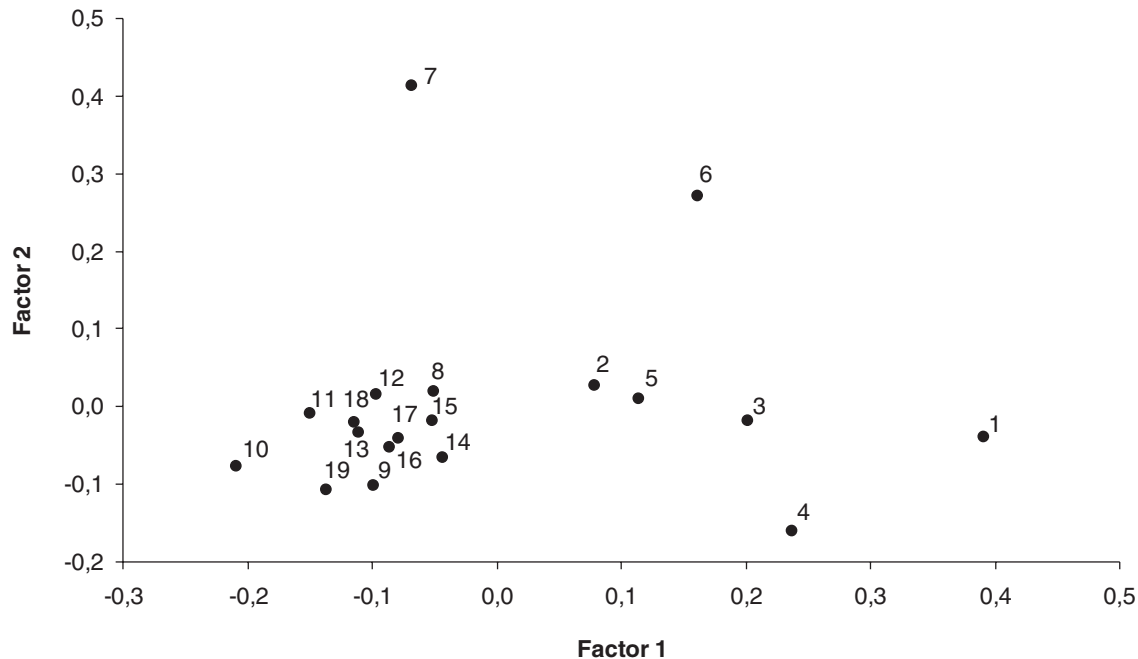


FIGURE 3. Results of a factorial correspondence analysis performed on allele frequencies of Atlantic cod in Greenland waters using 18 microsatellite loci along factors 1 and 2. See Table 1 for sample codes.

observed within groups, the most important information was the clear separation of the inshore and offshore samples (Figure 3). However, the offshore samples located in the northwest of Greenland (samples 6 and 7) clustered separately from the offshore samples on the second axis, suggesting that three genetic groups could be detected, although further investigation will be needed to confirm this result.

The locus-by-locus AMOVA also confirmed that among-group differentiation was significant between samples collected at inshore and offshore locations (Table 4) and showed that only eight microsatellite loci contributed to the observed differentiation. As expected for a marker under selection, the highest differentiation level was observed for *Gmo34*. Excluding this locus from the analysis resulted in a significant mean  $F_{ST}$  of 0.0012, and in 33 significant  $F_{ST}$  pairwise comparisons out of 171 (after sequential Bonferroni correction) among which 27 were due to inshore–offshore comparisons (without sequential Bonferroni correction, 51 comparisons were significant among which 45 were between inshore and offshore samples).

The Bayesian approach implemented in the software STRUCTURE revealed the existence of two distinct populations among the collected samples (Tables 5, 6), namely, inshore and offshore.

A comparison of the level of genetic diversity among inshore and offshore components using the software FSTAT (Goudet 1995) revealed significant differences at the level of expected and observed heterozygosity (inshore component:  $H_o = 0.808$ ,  $H_e = 0.837$ ; offshore component:  $H_o = 0.787$ ,  $H_e = 0.819$ ;  $P = 0.003$  and  $0.003$ , respectively).

#### Differentiation among Samples at the *Pan I* Locus

The partitioning of the genetic variance at the *Pan I* locus among and within the 19 samples, as estimated by  $F$ -statistics, showed a mean significant  $F_{ST}$  value of 0.233 and a  $F_{IS}$  of 0.013. The standardized genetic differentiation measured as  $G'_{ST}$  ( $G'_{ST} = 0.420$ ) was fivefold higher than the value observed for microsatellite loci ( $G'_{ST} = 0.086$ ). After sequential Bonferroni correction, pairwise differentiation between populations yielded 77 significant comparisons out of 171, of which 69 were observed between inshore and offshore samples. Indeed, *Pan I*<sup>BB</sup> genotypes were present in relatively high proportions at offshore but not inshore areas (Figure 4). Pairwise comparisons of the temporal samples revealed that except from the sample collected in area NAFO 1B in 2003 (sample 1), which was significantly different from the sample collected in the same area in 2004 (sample 2), all replicates were genetically indistinguishable (Table 3).

The AMOVA analysis confirmed these results and revealed that the among-group differentiation was highly significant among the samples collected at inshore and offshore spawning locations (Table 7). However, a comparison of the level of genetic diversity among inshore and offshore samples using the software FSTAT (Goudet 1995) did not reveal any significant differences.

#### DISCUSSION

Our study primarily aimed to assess the genetic variability of Atlantic cod in Greenland waters by genetically comparing

TABLE 4. Locus-by-locus analysis of molecular variance among inshore and offshore group of Atlantic cod populations (AIOG;  $F_{CT}$ ), among samples within groups (ASWG;  $F_{SC}$ ), and within samples (WS;  $F_{ST}$ ). The loci for which the inshore–offshore group comparison was significant are indicated in bold;  $P < 0.001^{***}$ ,  $P < 0.01^{**}$ ,  $P < 0.05^{*}$ .

Locus	%AIOG	%ASWG	%WS	$F_{CT}$	$F_{SC}$	$F_{ST}$
<b>Gmo2</b>	0.188	0.086	99.727	0.002*	0.001	0.003
<i>Gmo8</i>	0.011	0.032	99.957	0.001	0.001	0.001
<b>Gmo19</b>	0.107	0.193	99.700	0.001*	0.002*	0.003**
<b>Gmo34</b>	6.807	0.558	92.634	0.068***	0.006**	0.074***
<i>Gmo37</i>	0.076	0.318	99.606	0.001	0.003*	0.004***
<i>Tch5</i>	0.058	0.101	99.841	0.001	0.001	0.001*
<b>Tch11</b>	0.172	0.181	99.647	0.002**	0.002**	0.004***
<b>Tch14</b>	0.070	0.014	99.916	0.001*	0.001	0.001
<i>Tch22</i>	−0.053	0.527	99.526	0.000	0.005*	0.005*
<i>PGmo38</i>	0.022	0.040	99.938	0.001	0.000	0.000
<i>PGmo49</i>	0.094	0.093	99.814	0.001	0.001	0.002
<b>PGmo61</b>	0.130	0.080	99.791	0.001*	0.001	0.002
<i>PGmo74</i>	0.0611	0.159	99.780	0.001	0.002*	0.002*
<i>PGmo87</i>	0.041	−0.147	100.11	0.000	−0.147	−0.001
<i>PGmo94</i>	0.044	−0.090	100.05	0.000	0.000	−0.001
<i>PGmo100</i>	−0.013	0.185	99.828	0.000	0.002	0.001
<b>PGmo124</b>	0.109	0.175	99.716	0.001*	0.002*	0.003*
<b>PGmo134</b>	0.182	0.021	99.797	0.002***	0.001	0.002
Total	0.230	0.110	99.670	0.002***	0.001***	0.003***

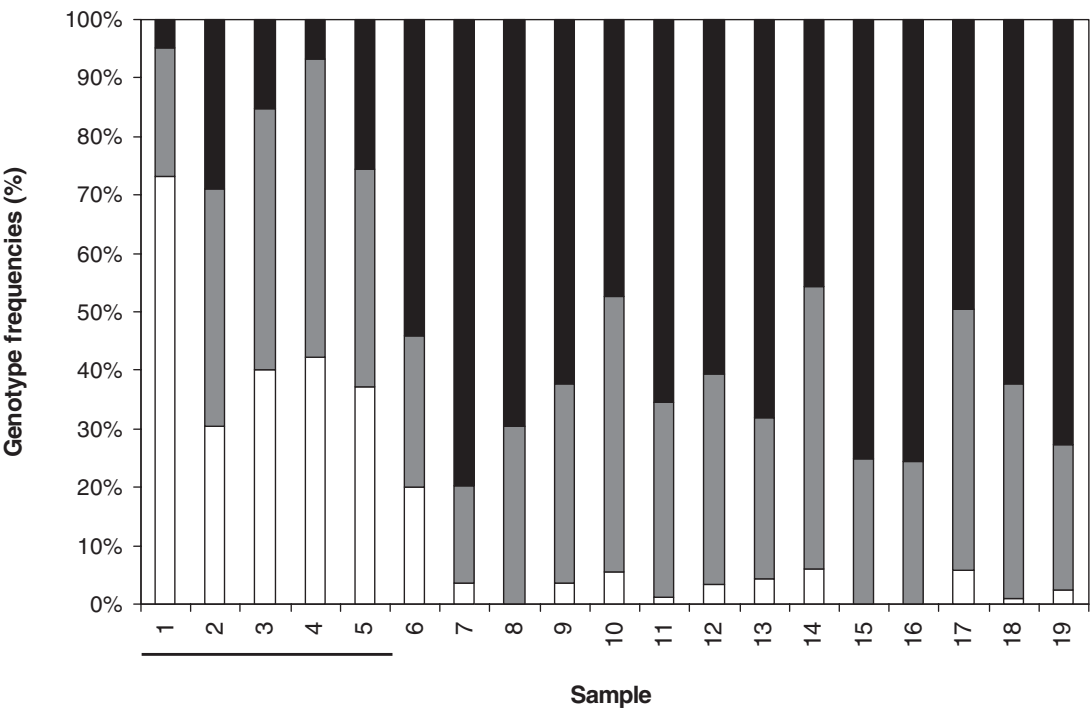


FIGURE 4. Distribution of genotypes of the *Pan* I locus in Atlantic cod from Greenland waters. Shading is as follows: solid bars =  $Pan\ I^{BB}$  genotypes; shaded bars =  $Pan\ I^{AB}$  genotypes; and open bars =  $Pan\ I^{AA}$  genotypes. Inshore samples (1–5) are indicated by a solid line under those numbers on the x-axis.

TABLE 5. Mean likelihood values (mean  $\log_e P[D]$ ) and SDs for  $K = 1$  to 7 after three runs of the program STRUCTURE (Pritchard et al. 2000). Bold italics indicate the most likely number of genetic cluster ( $K = 2$ ) contained in our samples.

$K$	Mean $\log_e P(D)$	SD
1	-130,495	107.10
2	<b>-129,658</b>	<b>264.70</b>
3	-130,809	413.83
4	-134,761	4,202.05
5	-136,396	3,996.85
6	-140,398	1,188.58
7	-145,177	5,860.49

TABLE 6. Mean admixture proportions ( $q$ ) and SDs of the 19 samples as assigned to the inshore cluster detected. Bold italics indicate the samples that constitute the inshore component.

Sample code	Mean $q$ for cluster 1	SD
<b>Inshore</b>		
1	<b>0.683</b>	0.105
2	<b>0.504</b>	0.122
3	<b>0.599</b>	0.108
4	<b>0.631</b>	0.102
5	<b>0.573</b>	0.113
<b>Offshore</b>		
6	<b>0.519</b>	0.124
7	0.443	0.118
8	0.455	0.105
9	0.447	0.103
10	0.376	0.098
11	0.434	0.119
12	0.406	0.117
13	0.361	0.124
14	0.475	0.114
15	0.464	0.120
16	0.451	0.116
17	0.442	0.103
18	0.436	0.119
19	0.458	0.120

local inshore and offshore populations collected from 2003 to 2005 in several NAFO areas and the ICES area XIVb in 2004 and 2006. Both types of genetic markers used during our study provided congruent results and suggested the presence of two distinct genetic components with limited connectivity in Greenland waters, namely an inshore and offshore component.

#### Genetic Variability within the Collected Samples

The genetic diversity of the 18 microsatellite loci (assessed as  $H_o$ ,  $H_e$ , allelic richness, and the mean number of alleles) exhibited a wide range of variation and was generally comparable with what has been observed in other marine fishes (DeWoody and Avise 2000). In addition, the offshore samples tended to exhibit a lower genetic diversity than their inshore counterparts, both at the microsatellite loci and the *Pan I* locus. This result might be due to the near extinction of the offshore populations 20 years ago, although such a difference in genetic diversity has already been described in several other geographical regions inhabited by Atlantic cod (Ruzzante et al. 1996; Pampoulie et al. 2006). Indeed, offshore populations, which usually occur at greater depths than their counterparts, appear to exhibit a lower genetic diversity (Ruzzante et al. 1996; Pampoulie et al. 2006). Another plausible explanation might be the replenishment of the offshore component by the inshore one, as recolonization of available habitat might result in a lower genetic diversity in the newly founded populations (Hewitt 1996; Pampoulie et al. 2008). Indeed, the genetic characteristics of samples 6 and 7 tend to support the latter hypothesis. At the microsatellite loci, these two samples were more closely related to the inshore samples from West Greenland, but exhibit *Pan I* genotypes that were similar to the offshore component. Therefore, the neutral markers seemed to support an inshore origin of these samples from cod that now dwell offshore and that seemed to have quickly adapted to the offshore conditions (see *Pan I* results). Although our study cannot clearly confirm this statement, such results suggest that inshore stocks replenish depleted offshore populations and at the same time can adapt to the new deeper offshore environment in less than a decade.

#### Differentiation among Samples

Both neutral and nonneutral genetic markers clearly revealed the presence of two genetic components in Atlantic cod within Greenland waters, namely, an inshore component and an offshore component, although samples 6 and 7 could be considered as a third potential group.

TABLE 7. Hierarchical analysis of molecular variance at the *Pan I* locus within and among samples of Atlantic cod grouped into inshore and offshore samples.

Type of variation	df	Variance components	% Variation	Fixation index	P-value
Among groups	1	0.10132	36.03	CT = 0.3603	<0.000001
Among samples within groups	17	0.00705	2.51	SC = 0.0392	<0.000001
Within samples	2,911	0.17286	61.46	ST = 0.3853	<0.000001
Total	2,929	0.29327	100		

The observed level of differentiation with the 18 microsatellite loci was similar to the genetic divergence observed in several coastal areas such as Iceland (Pampoulie et al. 2006), Canada (Bentzen et al. 1996; Ruzzante et al. 1996, 1998; Beacham et al. 2002), and Norway (Karlsson and Mork 2005; Dahle et al. 2006). The temporal stability of most of the samples and the stability of the level of genetic divergence among samples collected in different areas in different years suggested that the structure identified is real (Waples 1998).

At the *Pan I* locus, *Pan I*<sup>AB</sup> and *Pan I*<sup>BB</sup> genotypes were mainly encountered in the offshore spawning grounds (samples 6–19). However, as expected for a genetic marker known to be under selection (Pogson and Mesa 2004), it showed a stronger genetic differentiation ( $F_{ST} = 0.269$ ) than did the 18 microsatellite loci. The observed level of genetic differentiation was nevertheless in accordance with findings at comparable geographical scales (Fevolden and Pogson 1997; Sarvas and Fevolden 2005a, 2005b; Pampoulie et al. 2006).

In general, the observed results also corroborate previous genetic analyses carried out on hemoglobin polymorphisms (*HbI*<sup>I</sup> allele) and transferrin gene (*Tf*<sup>C</sup> allele) in Greenlandic cod, which showed that the frequency of the *HbI*<sup>I</sup> allele was homogeneous among West Greenland samples and that the *Tf*<sup>C</sup> transferrin allele exhibited a lower frequency in West Greenland samples than in Cape Farewell (south Greenland) samples (de Ligny 1969).

### Comparing Neural and Nonneutral Genetic Markers: Evidence for Adaptive Divergence

Recently, comparison of the genetic variation at neutral loci (microsatellites) and the nonneutral *Pan I* locus has been shown to be useful in assessing the potential effects of selection in shaping the population structure of Atlantic cod (Pampoulie et al. 2006; Skarstein et al. 2007; Westgaard and Fevolden 2007). In Icelandic waters, both types of genetic markers revealed congruent results and clearly discriminated two spawning components located in the northeast and southwest regions of Iceland (Pampoulie et al. 2006). These results have been interpreted as possible evidence of adaptive selection at the *Pan I* locus, which will typically lead to a higher variation in allele frequencies than drift alone (Gavrilets 2003). In Norwegian waters, neutral microsatellite loci did not show any genetic pattern among the well-known northeastern Arctic cod (NEAC) and Norwegian coastal cod (NCC) subdivisions in Norwegian waters, while nonneutral microsatellite loci and the *Pan I* locus clearly segregated them into two groups (Skarstein et al. 2007; Westgaard and Fevolden 2007). The authors stated that the discrepancy between these types of markers could be due to their evolutionary history related to mutational mechanisms and to the degree of selective neutrality. Thus, the differences among NEAC and NCC subdivisions were consequently due to diversifying selection that was not reflected in the evolution of the neutral markers (Westgaard and Fevolden 2007).

In the present study, both types of genetic markers (microsatellite loci and the *Pan I* locus) gave congruent results and thus the hypothesis of panmixia of Atlantic cod in Greenland waters was rejected, although the microsatellite loci yielded a lower level of genetic differentiation than did the *Pan I* locus. One of the microsatellite loci (*Gmo34*) exhibited a higher level of differentiation and did not follow neutral expectations according to the coalescent-based simulation. However, the observed genetic pattern was not exclusively due to this marker as demonstrated by the locus-by-locus AMOVA. When *Gmo34* was removed from the analysis, the overall level of genetic differentiation with neutral microsatellite loci was similar (0.0012) and still highly significant. Several recent studies demonstrated the nonneutrality of microsatellite loci in Atlantic cod, namely the locus *Gmo132* (Nielsen et al. 2006; Skarstein et al. 2007) and, soon after, *Gmo34* (Westgaard and Fevolden 2007). The standardized genetic measure ( $G'_{ST}$ ), which represents the proportion of maximum differentiation possible for the observed level of subpopulation homozygosity, revealed that the level of genetic differentiation is far more pronounced with the *Pan I* locus than with the 18 microsatellite loci. As suggested above, selection will typically lead to variation in allele frequencies in a shorter time than will drift alone, and thus seems to have resulted in a higher level of differentiation at the *Pan I* locus than that observed for neutral markers over a similar time-scale evolution. Therefore, we believe that these results suggest that divergent selection is acting on the *Pan I* locus and that restricted gene flow among the inshore and offshore Greenland cod populations (corroborated by the microsatellite loci results) contributes to the maintenance of the *Pan I* genetic pattern.

In general, it has been agreed that the genetic pattern of studied species is not altered by the inclusion of one or two nonneutral microsatellite loci (Nielsen et al. 2006) and that the combination of putatively neutral and nonneutral markers could convincingly reveal adaptive divergence with high (Hemmer-Hansen et al. 2007) or restricted (Pampoulie et al. 2006) gene flow (see Zane 2007 and Hauser and Carvalho 2008). Although adaptive divergence depends on the relative strength of gene flow and selection, it might be useful to combine several types of genetic markers to fully fathom the genetic structure of marine organisms and potentially integrate the observed results into fisheries management.

### Biological Implications and Fisheries Management

Recent biological and modeling studies indicated that Atlantic cod recruitment in offshore areas of Greenland waters was probably linked to the recruitment of the Iceland cod stock (Wieland and Hovgård 2002; Stein and Borovkov 2004; Storr-Paulsen et al. 2004; Ratz and Lloret 2005). In addition, the inshore cod populations of West Greenland were suggested to be separated entities that retained their genetic integrity despite the stock collapse, although occasional influx from offshore areas might occur (Storr-Paulsen et al. 2004). Therefore, these two hypothetical biological units have been managed as

different fisheries entities. In terms of spawning stock biomass (SSB), the offshore components in West and East Greenland waters almost disappeared 20 years ago (Storr-Paulsen et al. 2004; ICES 2010), while small sustainable populations were maintained in inshore areas. Here, the observed genetic pattern suggesting that inshore and offshore spawning locations comprise distinct stocks with limited connectivity (or medium connectivity with strong local selection) confirmed the biological information retrieved recently. However, further genetic analyses are required to fully understand the differentiation process within Greenlandic waters, e.g., a comparison of Greenlandic and Icelandic genetic variability to assess the origin of offshore Greenlandic cod. Most of the samples collected during the present study were composed of nonspawning fish that presumably originated from the Icelandic cod stock or from a mixture of Greenlandic and Icelandic cod. The lack of spawning events in Greenland waters further suggests that the fisheries might exploit feeding aggregations of presumably mixed origin.

The identification of stock structure has been widely recognized as a prerequisite for sustainable management of marine fisheries (Reiss et al. 2009). Indeed, the decline of most exploited species has now been attributed to a potential mismatch between biological and management units (see Reiss et al. 2009 for a review). Several genetic studies have investigated stock structure of exploited species within management areas and revealed significant genetic differences among inshore and offshore populations (Ruzzante et al. 1996; Pampoulie et al. 2006; Hyde et al. 2008; Stefánsson et al. 2009a, 2009b). Despite the plethora of genetic evidence for structure with depth (inshore versus offshore), fisheries units are still defined on a geographical basis. Although technical problems might emerge for a sustainable management of resources with depth, alternative solutions have been proposed (Cadrin et al. 2010). Indeed, geographical units might be defined according to depth and fisheries data as proposed for the beaked redfish *Sebastes mentella* (Cadrin et al. 2010). Currently, the Atlantic cod stock in Greenland waters is managed as two different units (ICES 1996): an offshore unit composed of East and West Greenland offshore populations and an inshore unit comprising inshore populations from West Greenland (NAFO Areas 1A to 1F). Until 2009, the inshore stocks have been subjected to catch constraints (ICES 2010). All available information indicates that the cod biomass in waters around Greenland is low and that the offshore component has been severely depleted since 1990 (ICES 2010). In recent years, concentrations of large, spawning Atlantic cod have only been found off East Greenland. The source of these spawning cod is presently unknown, but they may be an important element in the potential recovery of Atlantic cod in Greenland waters.

## CONCLUSION

The present study investigated the genetic structure of Atlantic cod in Greenland waters for the first time using neutral

and nonneutral genetic markers. Both types of genetic markers clearly indicate that the cod stock around Greenland is structured genetically into two major components, inshore and offshore. The comparison of neutral and nonneutral microsatellite loci plus the *Pan I* locus gave better insight into the potential effect of selection and led us to conclude that divergent selection associated with restricted gene flow were responsible for the maintenance of the observed genetic differentiation. Together with the additional recent genetic studies investigating the genetic variation in wild Atlantic cod populations using nonneutral and neutral genetic markers, the present study confirmed the usefulness of combining different genetic markers to reveal the potential effect of selection and gene flow on the observed genetic pattern.

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